THE HEMOGLOBIN OF THE BIVALVED MOLLUSC, PHACOIDES PECTINATUS GMELIN

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Hemoglobin has been reported from only a few species of bivalved molluscs; it occurs in cells in the blood of *Poromya granulata, Solen legumen, Tellina planata, Capsa fragilis, Cardita aculcata, Arca tetragona, Arca noae, Pectunculus glycimeris, Astarte fusca* (?) Griesbach, 1891), *Cardita sulcata* (Paladino, 1909), *Anadara inflata* (Kawamoto, 1928), *Arca pexata* (Svedberg and Hedenius, 1934) and *Arca subcrenata* (Kobayashi, 1935). In *Tivela stultorum* hemoglobin occurs in the brain (*sic*), mantle, gills, foot and adductor muscle (Fox, 1953).

Studies of absorption spectra have been carried out on the hemoglobin of *Arca subcrenata* by Kobayashi (1935), and Svedberg and Hedenius (1934) have run ultracentrifuge sedimentation studies on the pigment of *Arca pexata;* biochemically, however, the most extensively studied lamellibranch hemoglobin is that of *Anadara inflata.*

The study of *Anadara inflata* hemoglobin began with the work of Kawamoto (1928), who reported the oxygen dissociation curve of the pigment. Sato (1931) and Kobayashi (1935) established the absorption spectra of the pigment and its derivatives. Work on the hemoglobin of *Anadara inflata* culminated with the efforts of Yagi *et al.* (1955a, 1955b), who purified the hemoglobin, determined its molecular weight from sedimentation and diffusion studies, its electrophoretic mobility, iron content, nitrogen content and N- and C-terminal amino acids; in addition, molar extinction coefficients were also reported.

The present communication deals with the hemoglobin of the lucinid pelecypod, *Phacoides pectinatus* Gmelin, which the author chanced on in Puerto Rico. This animal lives deep (down to 18 inches) in the mud of mangrove swamps, and when it is opened exhibits dark purplish ctenidia with an appearance reminiscent of ripe muscatel grapes. The bloom on the surfaces of the ctenidia is due to a superficial layer of pigment-free cells; however, when the ctenidium is torn, it exhibits a bright red interior suggestive of hemoglobin. For illustration of the appearance of the gills see Figure 1. Although the clam is commonly eaten, at least in the neighborhood of La Parguera, P. R., and must therefore be somewhat well known, the author can find no mention of its hemoglobin in the literature.

A four-sided study of the red pigment of the ctenidia of *Phacoides pectinatus* has been undertaken. First, evidence for the identity of the pigment with hemoglobin has been obtained from studies of absorption spectra; second, the oxygen-combining properties of the pigment have been studied; third, the behavior of the pigment in the ultracentrifuge has been examined with a view to gaining some idea of the size of the molecule; and fourth, the histology of the pigment has been studied.

Materials and Methods

General

Phacoides pectinatus Gmelin was collected in the neighborhood of Point Pitahaya near La Parguera, Puerto Rico. They were stored at the Marine Station of the University of Puerto Rico on Magueyes Island, La Parguera, in running sea water at a temperature ranging between 26 and 28° C.; they were then taken back to the Biological Laboratories, Harvard University, where they were stored in aerated sea water at room temperature.

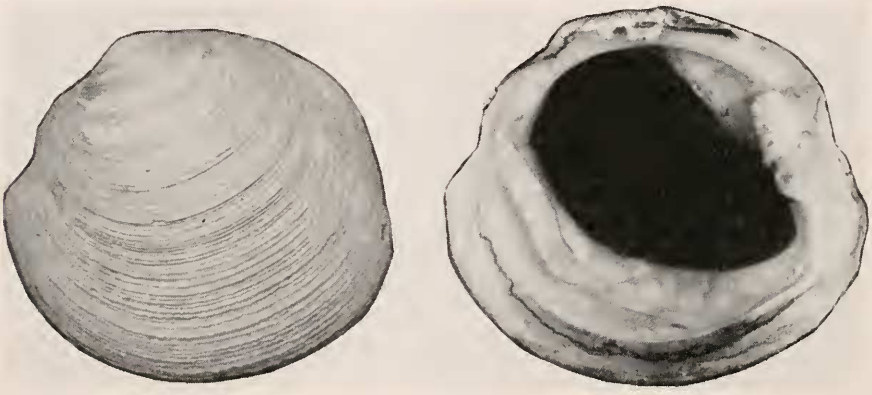

Figure 1. *Phacoides pectinatus* Gmelin. Length 5 cm. Dorsal side uppermost. The large dark mass shown by the dissected animal is the right ctenidium; the dark color is due to hemoglobin. Note the colorless edge of the demibranch, which is an indication of the hemoglobin-free, ciliated cells of the exterior of the gill. Members of the family Lucinidae have only the outer demibranch (Purchon, 1939).

Absorption spectra

Centrifuged homogenates of ctenidia, in phosphate buffers ranging in pH from 6.8 to 7.5 and molarity from 0.067 to 0.2 *M*, were examined spectroscopically with a Beckman Model DB recording spectrophotometer. Deoxygenation was brought about either by washing the preparation repeatedly with nitrogen or by adding a small amount of sodium dithionite. Carboxyhemoglobin was made by bubbling carbon monoxide through the dithionite-treated preparation or through the untreated preparation of oxyhemoglobin.

Oxygen dissociation curve

Samples of hemoglobin were prepared by homogenization of dissected ctenidia in a glass homogenizer with pH 7.4, 0.2 *M* phosphate buffer at 3° C. The homogenate was centrifuged at 8,000 × *g* for ten minutes to remove the coarser cellular debris, and the resulting supernatant was further centrifuged at 100,000 × *g* in the ultracentrifuge for 30 minutes.

After ultracentrifugation, the clarified preparation was transferred to tonometers of known volume, and the oxygen dissociation curve of the pigment determined by the method of Riggs (1951), slightly modified. In Riggs' method the solution

of hemoglobin in a tonometer, of which a cuvette for spectrophotometry forms an integral part, is freed of all oxygen by repeated washing with nitrogen; measured amounts of air are then introduced by way of syringes into the tonometer, and after each addition the absorption spectrum of the hemoglobin solution is measured. The oxygen tensions resulting from each increment of added air must be computed with regard being taken for the vapor pressure of water, oxygen dissolved in the water of the hemoglobin solution and oxygen combined with the hemoglobin itself; the degree of saturation of the hemoglobin with oxygen is computed from measurements of the absorption spectra at 560 mμ. The volume of the tonometers employed was about 430 ml. and the amount of hemoglobin solution 8 ml.; the amount of hemoglobin in solution was estimated from measurements of its optical density at the α peak and an assumed millimolar extinction coefficient of 15. The absorption spectra were measured with Beckman Model DB or Cary Model 11MS recording spectrophotometers. At the end of each run a check against leakage was automatically provided, since the actual pressure within a tonometer could be compared with the pressure computed from measurements of the initial pressure and the varying increments of added gas; the results were deemed acceptable if the pressures obtained by the two methods agreed within 1 mm. Hg.

Sedimentation constant

A sample of centrifuged homogenate of ctenidia in pH 7.4, 0.2 *M* phosphate buffer was placed in the cell of a Spinco Model E analytical ultracentrifuge and the sedimentation constant determined at 59,780 rpm with a Wratten No. 2412 filter (red) in the optical system.

The sedimentation constant s of a protein is the velocity of sedimentation of the protein molecule in a field of centrifugal force of unit strength under the particular conditions of medium and temperature at which the determination is performed. A correction factor can be estimated from tables given by Svedberg and Pedersen (1940), whereby the experimental value of s may be corrected for viscosity and density effects to a base of 20° C. and water; the value for the sedimentation constant under these conditions is given the symbol $s_{20,w}$.

As a protein sediments it leaves a volume of solution clear of that particular protein directly behind it so that a boundary is formed. The concentration gradient represented by the moving boundary is converted into a peak by an optical system and the position of this peak is photographed at intervals of time; this allows the rate of sedimentation of the protein to be followed. Now there may be several proteins in the sample, and if one of them is colored, a change of density of film exposure will be associated with the colored protein's peak, since along with the peak the contents of the sedimentation cell are also photographed. Thus, in Figure 3 if it is assumed that the only colored protein present is hemoglobin, then this will be the protein associated with the slow peak and as such it has been taken.

Histology

The ctenidial tissue of *Phacoides pectinatus* was treated in two ways. The first approach was the examination of unstained cryostat sections, while the second

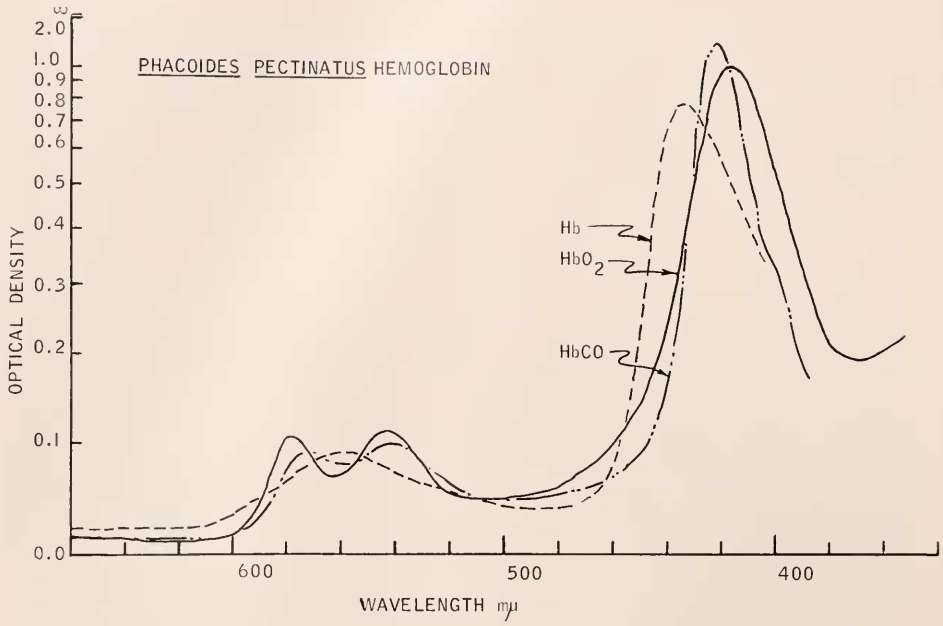

FIGURE 2. Absorption spectra of *Phacoides pectinatus* hemoglobin and derivatives.

included the fixation of the tissue in 10% acrolein in xylene at 0° C., embedding in wax and staining for occult iron by the methods of Glick and Hutchison as described in Humason (1962), somewhat modified.

The most satisfactory method for the iron stain proved to be the following: (1) Deceration and hydration of the tissue to water; (2) two minutes in 30% H_2O_2 alkalized with one drop concentrated NH_3 solution per 100 ml.; (3) thorough rinsing with several changes of distilled water for 5 minutes; (4) 15 minutes at 56° C. in a freshly made, filtered and heated solution of acidified potassium ferrocyanide, made by mixing 25 ml. each of solutions containing 2 g. potassium ferrocyanide to 50 ml. water and 2 ml. concentrated HCl to 50 ml. water; (5) thorough rinsing with several changes of distilled water for 5 minutes; (6) counterstaining 4 minutes in 1% carminic acid; (7) 5 minutes' differentiation in 4% potassium aluminum sulfate; (8) thorough rinsing in several changes of distilled water for 5 minutes; (9) dehydration, clearing and mounting. The iron-containing material is stained a bright green and the nuclei red; the remainder of the tissues stain various shades of pink.

RESULTS

Absorption spectra

The results of the measurement of the absorption spectra of the hemoglobin of *Phacoides pectinatus* are shown in Figure 2. In the following description the numbers in parentheses represent the range of the observations in mμ and the number of observations, respectively. The *Phacoides pectinatus* preparation in

the oxygenated state shows strong α, β and Soret bands with peaks at 578.7 (1,6), 543.3 (2,6) and 416.3 mμ (1,3), respectively; upon deoxygenation the α and β bands disappear, to be replaced by a single band with a peak at 558.1 mμ (2,6), while the Soret peak shifts to 433.5 mμ (1.2); treatment of the preparation with carbon monoxide causes reappearance of the α and β bands, but shifted towards the violet compared with oxyhemoglobin, and their peaks lie at 572 (0,2) and 541 mμ (0,2), respectively; the Soret band of the carbon monoxide-treated prepa-

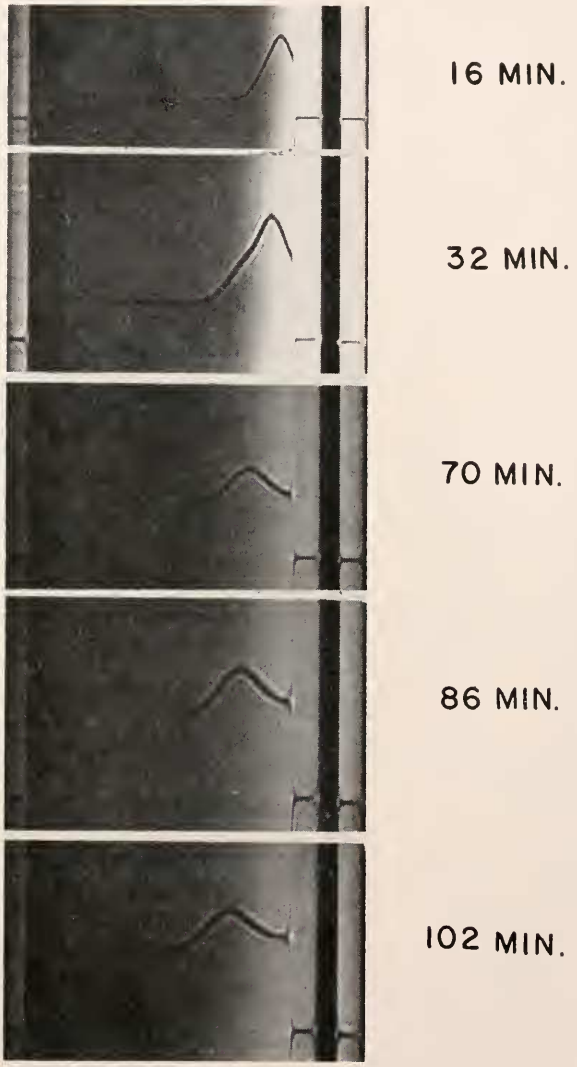

FIGURE 3. Ultracentrifuge run. The hemoglobin is sedimenting from right to left. Note how the larger schlieren peak is associated with the change in optical density in the cell, indicating that it is probably the hemoglobin peak. The 16- and 32-minute photographs were taken on a different plate from those taken during the interval 70 to 102 minutes.

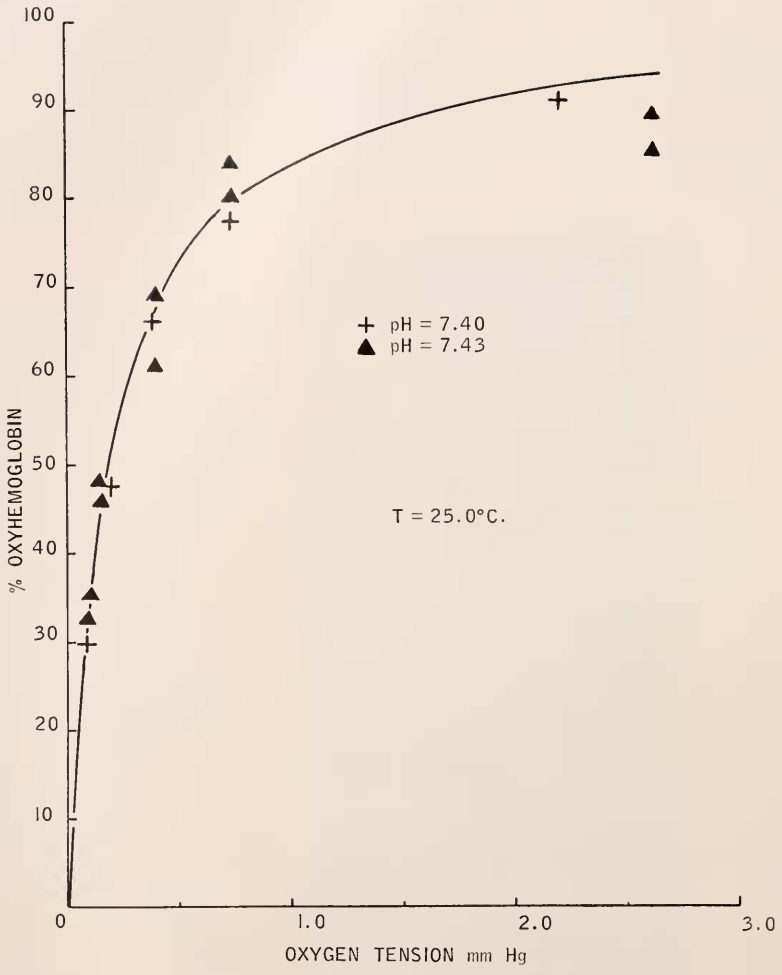

FIGURE 4. Oxygen dissociation curve for *Phacoides pectinatus* hemoglobin. The solid line is drawn for the Hill equation with p_{50} equal to 0.19 mm. Hg and n equal to 1.

ration shifts to 422 mμ (0,2). The *Phacoides pectinatus* preparation absorbs to a greater extent at the β peak than at the α for both oxy- and carboxyhemoglobin.

Oxygen dissociation curve

The results of this section are summarized by Figure 4. The curve shown is drawn from the well-known Hill Equation:

$$y = \frac{(p/p_{50})^n}{1 + (p/p_{50})^n}$$

with p_{50} equal to 0.19 mm. Hg and n equal to 1; y represents the fraction of hemoglobin in the oxygenated form, p the partial pressure of oxygen, p_{50} the

partial pressure of oxygen at 50% saturation of the pigment and n a constant which can be considered a measure of heme-heme interaction. A value of n equal to 1 indicates zero heme-heme interaction and a hyperbolic dissociation curve.

Sedimentation constant

Two very fast-moving peaks rapidly formed and swiftly moved across the field of view even before the ultracentrifuge had reached full speed. The hemoglobin peak then formed (see Figure 3) and this in turn split into two peaks, with the slower moving peak appearing to be associated with the hemoglobin, as judged by the optical density changes in the cell. The value of the sedimentation constant $s_{20,w}$ was found to be 2.2×10^{-13} seconds from one determination only.

Histology

Microscopic examination of the unstained cryostat sections showed highly localized patches of yellow-brown pigment scattered throughout the cells of the interior of the gill. The patches were in the form of dense granular masses about 5–7 μ in diameter, with the individual granules ranging from about 0.6 to 1.3 μ in diameter; this is apparent from the photomicrograph in Figure 5.

Examination of the tissue stained for occult iron showed green granular masses

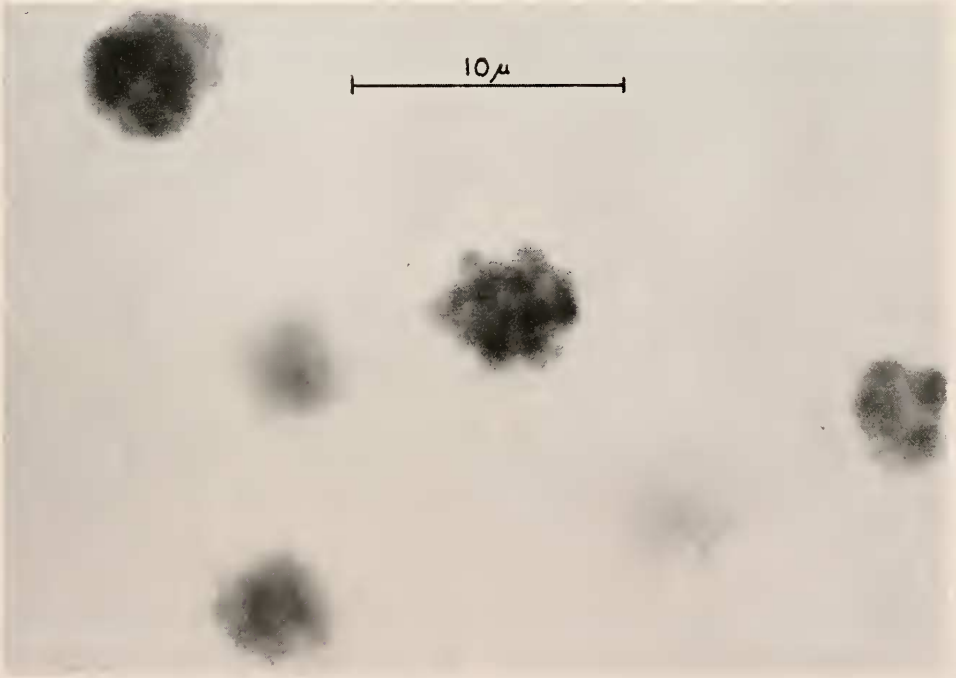

FIGURE 5. Photomicrograph of unstained cryostat section of the ctenidium of *Phacoides pectinatus* taken without a filter in the optical system. The color of the dark masses was yellow-brown.

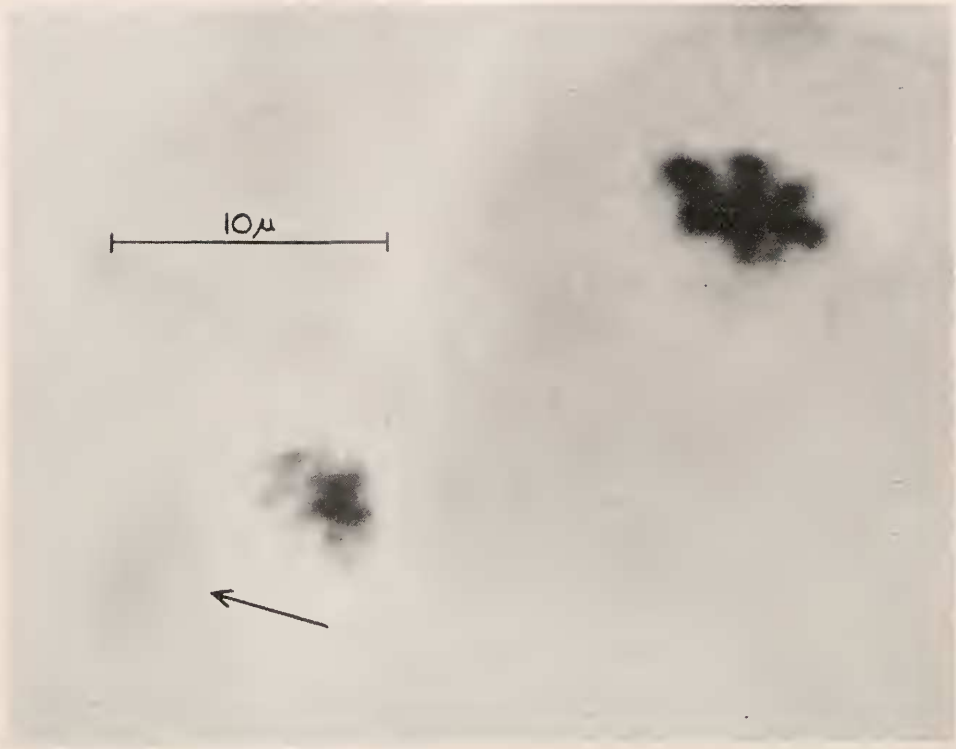

FIGURE 6. Photomicrograph of section stained for occult iron, taken with a Wratten No. 25 (red) filter in the optical system; the magnification is the same as for Figure 5. The color of the two dense granular masses was green; note the shadowy image of the nucleus at the left-hand side of the picture (arrow).

of approximately the same size and structure as the yellow-brown masses seen in the unstained cryostat section; Figure 6 shows a photomicrograph taken with a Wratten No. 25 (red) filter in the optical system at the same magnification as the photomicrograph of the unstained cryostat section in Figure 5.

The green-staining masses were confined to the interior cells of the gill and were not associated with the ciliated cells of the exterior surface of the ctenidium; this distribution is apparent from examination of Figures 7a, b and c, which are photomicrographs of the same section of the ctenidium taken without any filter, with a Wratten No. 58 (green) and with a Wratten No. 25 (red) filter in the optical system, respectively.

DISCUSSION

Absorption spectra

The behavior of the *Phacoides pectinatus* preparation, with respect to its absorption spectra under various conditions, is very strong evidence in favor of its containing hemoglobin. Unfortunately, the absorption spectra of other lamellibranch hemoglobins appear to be known only for *Arca inflata, Arca subcrenata* and *Tivela stultorum*.

For *Arca inflata* the following wave-lengths for the absorption maxima have been reported: oxyhemoglobin α peak, 578 mμ (Sato, 1931; Kobayashi, 1935), 576 mμ for the purified preparation of Yagi *et al.* (1955a); oxyhemoglobin β peak, 540.8 mμ (Sato, 1931), 541 mμ (Kobayashi, 1935), 540 mμ (Yagi *et al.*, 1955a); deoxyhemoglobin, 559 mμ (Sato, 1931), 556 mμ (Kobayashi, 1935), 555 mμ (Yagi *et al.*, 1955a); carboxyhemoglobin α peak, 573.1 mμ (Sato, 1931), 570 mμ (Yagi *et al.*, 1955a); carboxyhemoglobin β peak, 537.8 mμ (Sato, 1931), 540 mμ (Yagi *et al.*, 1955a).

For *Arca subcrenata*, Kobayashi (1935) obtained values for the wave-lengths of the absorption peaks identical with those for *Arca inflata*. Fox (1953) states that the hemoglobin of *Tivela stultorum* shows sharp absorption bands at 577 and 540 mμ.

With regard to the extent of the absorption at the α and β peaks of oxyhemoglobin, Kobayashi (1935) found that the ratio of the absorption of the α peak to that at the β peak was 0.99±0.02 for *Arca inflata* (?) and 1.00±0.05 for *Arca subcrenata;* Yagi *et al.* (1955a) with a purified preparation of *Anadara inflata* hemoglobin observed values of the millimolar extinction coefficient, ϵ_{mM}, for the α peak of 11.1 and for the β peak of 11.0, thus indicating a slightly greater absorption at the α peak than at the β; this difference was intensified when the carboxyhemoglobin was examined, and here the value of ϵ_{mM} for the α peak was 11.2 and for the β peak 10.8.

The hemoglobin of *Anadara inflata* thus has absorption peaks at wave-lengths only slightly different from those obtained for *Phacoides pectinatus*. However, the two species differ in the values they show for the ratio of absorbency at the α peak to that at the β peak, both for oxy- and carboxyhemoglobin; for *Phacoides pectinatus* this value is slightly less than one, while for *Anadara inflata* the reverse is true.

Oxygen dissociation curve

Manwell (1960a) and Prosser and Brown (1961) have thoroughly reviewed the subject of the function of invertebrate respiratory pigments. In order for a respiratory pigment to play any role in the transport of oxygen from the environment to the tissues, the oxygen tension at which the pigment becomes saturated with oxygen must be of at most the same order of size as the partial pressure of oxygen in the environment; this statement is supported by the innumerable cases in which the oxygen affinities of invertebrate respiratory pigments have been correlated with the ecology of the species studied, such that high oxygen affinities are associated with low environmental oxygen tensions. A state of controversy exists as to whether the pigments serve as reserve stores of oxygen, which come into use only when ambient oxygen levels become low, or whether they serve to facilitate the transfer of oxygen, even when ambient oxygen levels are high enough to maintain the pigment almost entirely in the saturated condition. Those who hold the latter view, point out that the total oxygen capacity of the pigment is too small to constitute an effective reserve. In actuality the pigment probably serves both purposes, facilitating oxygen transport even when environmental oxygen tensions are high and acting as a small reserve store when low, except in the nematodes where the function of their hemoglobin is not understood.

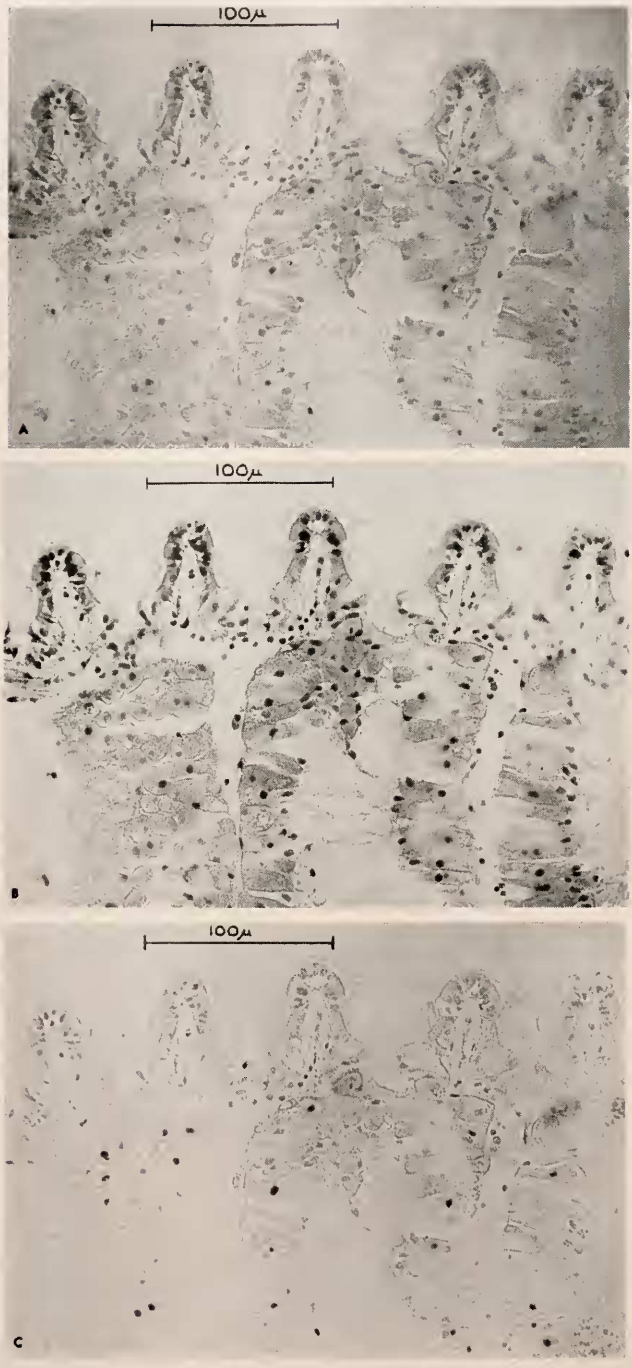

FIGURE 7a. Photomicrograph of section of gill stained for occult iron, taken with no filter in the optical system. Note the dense mats of cilia of the ciliated cells of the surface of the ctenidium.

The oxygen affinity of at least one lamellibranch hemoglobin, that of *Anadara inflata,* has hitherto been determined; working with hemolyzed blood of this species, Kawamoto (1928) found a value for p_{50} equal to 10 mm. Hg at 20± 1° C. and a value for n of 1.155; the determination was made in the absence of CO_2.

Species of *Anadara* are surface-living forms (as far as this author is aware); *Phacoides pectinatus,* on the other hand, lives deep in what are most likely anaerobic muds. The differences in the values of p_{50} reflect the differences in ecology of the two species. The hemoglobin of *Phacoides pectinatus* with its p_{50} of 0.19 mm. Hg is far more comparable to that of the polychaete worm, *Travisia pupa* than to *Anadara inflata.* This worm was studied by Manwell (1960b), and its muscle hemoglobin has a p_{50} of only 0.08 mm. Hg; coelomic hemoglobin, 0.36 mm. Hg; and vascular hemoglobin, a p_{50} varying between 0.53 and 1.10 mm. Hg, depending on pH; this worm lives, like *Phacoides pectinatus,* totally buried in the mud and apparently dies of oxygen poisoning when removed from its substrate and kept in aerated sea water. However, in making interpretations such as these from values of p_{50} or n, obtained from impure or unphysiological preparations of a respiratory pigment, it must be borne in mind that the values for these same parameters obtained for the pure pigment or for the pigment in the intact animal may be different. This was brought out by Manwell (1960c) who showed how values of n changed with the degree of manipulation of the pigment.

Sedimentation constant

The results of the sedimentation determination should be taken with certain reservations, not only because of the impure nature of the preparation, but also because the results have not been extrapolated to zero protein concentration; the possibility also exists that actually it may not have been the hemoglobin peak which was measured.

Prosser and Brown (1961) have published a table giving values of molecular weight and $s_{20,w}$ for a number of respiratory proteins. Vertebrate hemoglobins, excluding those of the cyclostomes, contain four heme groups and associated polypeptide chains, and have molecular weights of about 4 × 17,000; values of $s_{20,w}$ obtained for this type of molecule are about 4.5 × 10^{-13} seconds. The circulating hemoglobins of the cyclostomes appear to have but one heme group per molecule, molecular weights ranging from 19,100 to 23,100 and values of $s_{20,w}$ ranging from 1.87 to 2.3 × 10^{-13} seconds.

Sedimentation constants have been measured for the hemoglobins of two other species of lamellibranch. Svedberg and Hedenius (1934) reported values of $s_{20,w}$ ranging between 3.20 and 4.09, with a mean of 3.46 × 10^{-13} seconds, for the hemoglobin of *Arca pexata* in an impure preparation. Yagi *et al.* (1955a) reported a value of $s_{20,w}$ of 4.6 × 10^{-13} seconds at pH 7.45 for their purified preparation of

FIGURE 7b. Same section as Figure 7a, taken with a Wratten No. 58 (green) filter in the optical system. This filter accentuates the red-staining nuclei and pink background in relation to the green-staining iron-containing granules.

FIGURE 7c. Same section as in Figures 7a and 7b but taken with a Wratten No. 25 (red) filter in the optical system. This filter accentuates the green-staining iron-containing granules. Note, by comparing Figures 7a, 7b and 7c, how the green-staining granules are confined to the cells of the interior of the ctenidium and are not associated at all with the cells of the surface of the ctenidium; compare this with the legend under Figure 1.

Anadara inflata hemoglobin; these data were combined with measurements of the diffusion constant to give a molecular weight of 71,000; coupled with data on iron content, which was about 0.31%, this suggests that molecules of *Anadara inflata* hemoglobin are of a size and shape similar to those of the hemoglobins of higher vertebrates.

The value of $s_{20,w}$ of 2.2×10^{-13} seconds obtained for the hemoglobin of *Phacoides pectinatus* suggests that the molecule consists of one heme-polypeptide unit with a molecular weight in the neighborhood of 17,000. The apparent absence of heme-heme interactions in the oxygen dissociation curve is consistent with a molecule that contains but one heme group; in addition, the apparently low molecular weight is consonant with the intracellular location of the pigment (Prosser and Brown, 1961).

Histology

Two-fold lines of evidence are presented for the occurrence of hemoglobin in the form of granular masses within the cells of the interior of the ctenidia of *Phacoides pectinatus*. First, the color of the granules in the cryostat sections strongly suggests hemoglobin, and second, particles of similar size, shape, and structure take the characteristic green color of iron with the ferrocyanide stain.

One of the most striking findings emerging from the histological studies is the fact that the hemoglobin, if such the yellow- or green-staining particles be, does not appear to be associated with the energy-using ciliated cells of the surface of the ctenidium. The localization of the pigment in granules within the cells is similar to the findings of Griesbach (1891) and Sato (1931) for the erythrocytes of certain other lamellibranchs.

The localization of the presumptive hemoglobin within the cell is in accord with its sedimentation characteristics, which indicate that it has low molecular weight, perhaps of the order of 17,000, and would tend to escape by diffusion unless it were confined within a membrane.

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Summary

1. The ctenidia of *Phacoides pectinatus* contain hemoglobin.

2. The hemoglobin appears to be located in the inner tissue of the ctenidia; it does not appear to be associated with the ciliated cells of the surface of the ctenidia.

3. In the cells in which it occurs, the presumptive hemoglobin is highly localized in the form of granular masses, of diameter about 5–7 μ, with the individual granules having diameters ranging from about 0.6 to 1.3 μ.

4. Evidence is presented that the hemoglobin has a sedimentation constant $s_{20,w}$ of 2.2×10^{-13} seconds, but this value needs confirmation.

5. The oxygen dissociation curve of the hemoglobin exhibits no evidence of heme-heme interaction. The pigment has a p_{50} of 0.19 mm. Hg at 25° C. in pH 7.4, 0.2 *M* phosphate buffer in an impure preparation; this value is in accord with the ecology of the species.

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